P[illegible]TERONE PRODUCTION BY ENZYMATICALLY DISPERSED CELLS FROM CORPORA LUTEA OF THE SPINY DOGFISH, SQUALUS ACANTHIAS. Lorrie L. Klosterman and Ian P. Callard, Department of Biology, Boston University, MA 02215

The lengthy gestation period of the spiny dogfish (20-22 months; Hisaw and Albert, Biol. Bull., Marine Biol. Lab. Woods Hole 92:187-199, 1947) provokes the question of whether progesterone synthesis by the ovary, and specifically the postovulatory follicle (corpus luteum), is physiologically important in maintaining pregnancy in this species. Minced corpora lutea of the dogfish under pituitary control synthesize progesterone in vitro (Tsang and Callard, J. Exp. Zool, in press). To further address the issue of the regulation of luteal function, we devised a method by which to obtain a single-cell suspension of luteal cells to facilitate in vitro studies. We describe here a method for the enzymatic dispersion of luteal cells using collagenase, and present the results of preliminary experiments using this method in which progesterone production by luteal cells was greatly enhanced by substrate (25-OH-cholesterol) supplementation.

Ovaries were obtained from pregnant dogfish in gestational stages A and C (in early first and second year of pregnancy, respectively; Hisaw and Albert, Biol. Bull., Marine Biol. Lab. Woods Hole 92:187-199, 1947) and placed in ice-cold elasmobranch buffer (Forster, Goldstein, and Rosen, Comp. Biochem. Physiol. 42A:3-12, 1972) immediately after animals were immobilized by pithing. Ovarian weight and the number and size of follicles were recorded. Maximum ovarian follicular diameter and length of pups were used to classify females into gestational stages. Corpora lutea were dissected out, individually weighed, pooled according to stage, and scissor-minced in ice-cold buffer. Minces were washed three times in buffer and resuspended in Eagle's Basal Medium (BME) containing 5 mM glucose, 350 mM urea, 0.1% collagenase (Type IV; Sigma), 0.05% DNase, and 0.01% sodium heparin. Tissues were further minced and teased with forceps in this mixture at room temperature, then moved to a 50 ml culture tube placed in a near-horizontal position. A stream of 95% O_2/5% CO_2 was directed at the surface of the liquid, which resulted in a mild vortex affect that allowed simultaneous oxygenation and stirring of the mixture. After 1-2 hrs of collagenase treatment at room temperature, the mixture was filtered through a fine mesh (60-80) and cells harvested by centrifugation of the filtrate at 800g. Cells were resuspended in 1 ml BME containing glucose, urea, and 0.01% sodium heparin (the latter prevented rapid re-aggregation of cells). Trypan blue exclusion indicated about 70% of cells were viabile after this procedure (compared to almost no recovery of intact cells after treatment with trypsins). Cells in the suspension were counted using a hemocytometer and the volume of culture medium adjusted to deliver 1-2 X 10^5 cells in 0.1 ml. Aliquots of 0.1 ml were dispensed in triplicate into glass test tubes or 24-well culture plates, and a final incubation volume of 0.5 ml was obtained by adding medium alone (controls) or medium containing the following, alone or in combination: 1) an aqueous extract of the gonadotropin-containing ventral lobe of the pituitary, 2) the relatively polar metabolic precursor of progesterone, 25-OH-cholesterol (in a range of final concentrations from 10 ng/ml to 2 ug/ml), or 3) 5% Squalus serum, previously stripped of endogenous steroids by incubation with 2.5% dextran-coated charcoal for 1 hr at room temperature. Incubations were carried out at 18-20° C for 18-48 hrs in an atmosphere of O_2/CO_2. Incubations were terminated by centrifugation to separate cells; the medium was poured off and frozen for subsequent measurement of progesterone content by a radioimmunoassay previously validated in our laboratory, and cell pellets were frozen for subsequent protein determination.

The collagenase/DNase dispersion resulted in yields of 1-4 million viable cells per gram of luteal tissue. Initial experiments demonstrated the ability of cells to synthesize progesterone *in vitro*, in quantities ranging from 115 ± 6 to 1,520 ± 270 pg/ml/100,000 cells in 18 hrs, independent of gestational stage. However, in some experiments hormone production was negligible, and the accumulation of lipid in the medium during collagenase treatment often was noted. Since isolated cells in culture may suffer reduced and variable intracellular stores of substrate (cholesterol) which are neccessary for steroid synthesis, in subsequent experiments 25-OH-cholesterol was added to the incubation medium. Initial experiments using stage A luteal cells showed that at least 80 ng/ml substrate was required for enhanced production at a cell density of about 100,000 cells/ml (Fig. 1). A concentration of 2 ug/ml dramatically stimulated hormone production by stage C luteal cells over controls (Figure 2). The addition of 5% charcoal-stripped *Squalus* serum appeared to have an additive effect on this stimulation.

The influence of homologous pituitary (aqueous extract equivalent to 1/10 of the ventral lobe) had relatively little (in stage C) or no (stage A) effect on progesterone production (Figures 1 and 2). Recent experiments using luteal cells from other stages (B and D) likewise showed no stimulatory effect of pituitary extract in concentrations equivalent to 1/40, 1/20, or 1/10 pituitary (data not shown).

These results demonstrate that isolated cells from the corpus luteum of the dogfish are capable of progesterone synthesis. The occasional absence of steroidogenesis by dispersed cells (regardless of gestational stage), and its marked enhancement by the addition of 25-OH-cholesterol, suggest that cellular stores of cholesterol may be reduced during the procedure to a level below that neccessary for detectable hormone synthesis. In fact, addition of substrate consistently increased progesterone output for all gestational stages. In contrast, increased hormone production in response to pituitary extract is only evident at stage C, an observation made previously in experiments using luteal pieces (Tsang and Callard, J. Exp. Zool., in press). Thus, the ability of substrate alone to enhance steroidogenesis *in vitro* far exceeds that of the pituitary factors. However, the further enhancement of hormone output by stage C cells in the presence of serum may be due to gonadotropins. In combination, these studies suggest that luteal cells are competent to produce progesterone during most of gestation without pituitary stimulation, if substrate availability is not limiting. This does not mean that luteal steroidogenesis *in vivo* is without pituitary regulation. A control mechanism would in fact be required if hormone production were to be modulated. Whether such control is effected by the pituitary or by intracellular cholesterol availability remains to be evaluated.

This research was supported by NSF DCB 85-18296 to I. P. Callard.

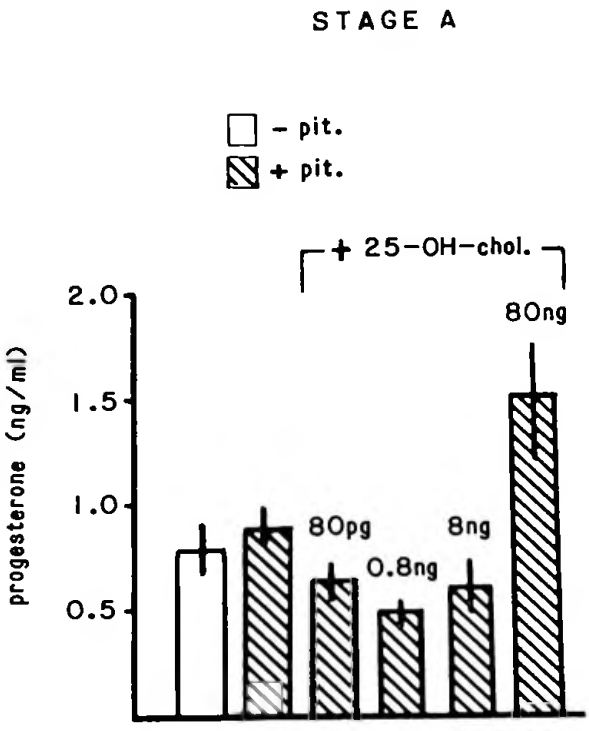

FIGURE 1. Progesterone production (X ± S.E. of triplicate tubes) by stage A luteal cells in the presence or absence of 25-OH-cholesterol and pituitary extract. Incubation time, 18 hr.

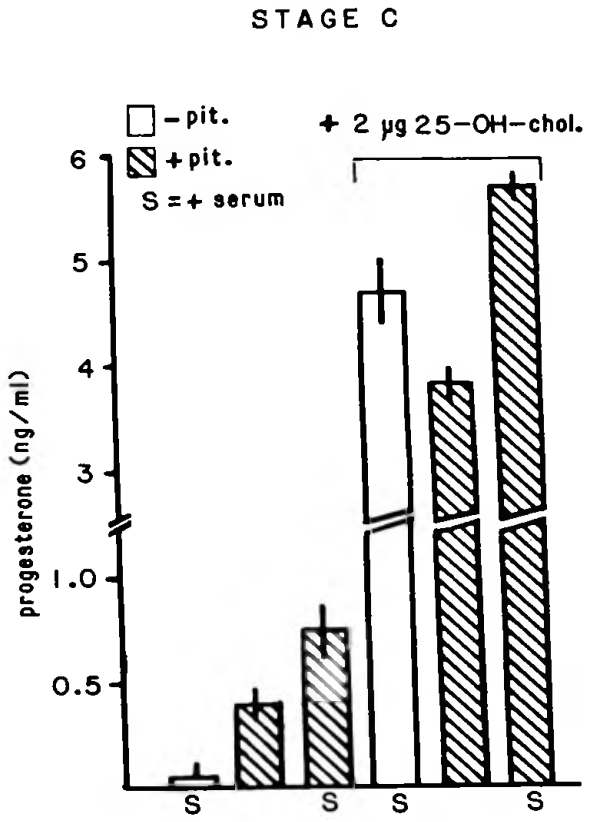

FIGURE 2. Progesterone production (X ± S.E. of triplicate tubes) by stage C luteal cells in the presence or absence of 25-OH-cholesterol, pituitary extract, and homologous serum. Incubation time, 18 hr.